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DOI: https://doi.org/10.1016/j.diagmicrobio.2017.09.014

Diagnostic Microbiology and Infectious Disease xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

A novel RT-PCR for the detection of *Helicobacter pylori* and identification of clarithromycin resistance mediated by mutations in the 23S rRNA gene

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ARTICLE INFO

Article history:
Received 17 July 2017
Received in revised form 21 September 2017
Accepted 22 September 2017
Available online xxxx

Keywords:
Helicobacter pylori detection
Clarithromycin resistance identification
RT-PCR

ABSTRACT

In this study we evaluated the commercially available LightMix® RT-PCR assay for *Helicobacter pylori* detection and identification of clarithromycin (CLR) resistance in culture and clinical specimens (gastric biopsies and stool). The *H. pylori* LightMix® RT-PCR detects a 97 bp long fragment of the 23S rRNA gene and allows the identification of 3 distinct point mutations conferring CLR resistance via melting curve analysis. The performance of the *H. pylori* LightMix® RT-PCR was evaluated using a set of 60 *H. pylori* strains showing phenotypical CLR susceptibility or CLR resistance (Minimum inhibitory concentrations from 0.016 to 256 mg/L). We found high concordance (95%) between phenotypical CLR resistance screening by E-Test® and the Lightmix® RT-PCR. Discrepant results were verified by sequencing of the 23S rRNA gene that always confirmed the results obtained by Lightmix® RT-PCR. Furthermore, *H. pylori* was detected in clinical biopsy and stool specimens by Lightmix® RT-PCR that identified the correct *H. pylori* genotype. The LightMix® RT-PCR is an accurate, sensitive and easy to use test for *H. pylori* and CLR resistance detection and can therefore be readily implemented in any diagnostic laboratory.

1. Introduction

Helicobacter pylori is a Gram-negative bacterium that can cause chronic stomach infections and is therefore known as the main cause of gastric ulcers and subsequently for the development of gastric cancer if untreated (Thaker et al., 2016). Therefore, eradication therapy of *H. pylori* is necessary in order to prevent the development of gastric adenocarcinoma (Fukase et al., 2008) as well as for the successful treatment of mucosa-associated lymphoid tissue (MALT) lymphoma (Fischbach et al., 2004). The proposed first-line empirical treatment for an *H. pylori* infection is a triple therapy including a proton pump inhibitor (PPI), clarithromycin (CLR) and amoxicillin or metronidazole (Malfertheiner et al., 2017). Over the past 20 years increasing resistance rates of *H. pylori* to macrolides such as CLR have been reported (Megraud et al., 2011), with a prevalence of CLR resistance in Europe at present around 19% (Megraud et al., 2013). The increase in CLR resistance impairs the effectiveness of empiric triple regimens (Fischbach et al., 2002). As a result a vast majority of patients show unacceptably low treatment success for first-line empirical treatment containing CLR, with only 18% exceeding 85% and approximately 60% failing to reach 80% eradication rates (Graham and Fischbach, 2010). Consequently, empiric triple therapy is just recommended in regions with CLR resistance rates lower 20% without prior susceptibility testing (Malfertheiner et al., 2017). Molecular methods, like RT-PCR, can be used to determine CLR resistance before the start of empiric therapy and can achieve increased *H. pylori* eradication rates by individually adapted antibiotic therapy. As second line therapy for *H. pylori* eradication, either a bismuth containing quadruple therapy or a levofloxacin containing triple therapy is recommended (Malfertheiner et al., 2017; Mégraud, 2013).

In Switzerland, the current algorithm for *H. pylori* detection is to perform an endoscopy, from which biopsy-based tests such as rapid urease screening, histology and culture are done (Malfertheiner et al., 2017). Susceptibility testing from culture is recommended to ensure the best choice of antibiotics (Cammarota et al., 2004), especially after treatment failure with first-line and second-line antibiotics. In times of increasing antibiotic resistance, the need for more rapid resistance screening procedures that replace conventional cultural methods gain importance as *H. pylori* is a fastidious and slow growing bacterium that can make susceptibility testing from culture a time consuming and challenging task.

Here, we introduce the commercially available Lightmix® RT-PCR method that is based on the detection of point mutations in the gene encoding the 23S ribosomal subunit (23S rDNA) of *H. pylori*. There are three main point mutations in the 23S rDNA (A2142G, A2143G and A2142C) that are known to induce macrolide resistance. It is possible to distinguish 3 types of point mutations from the wild-type sequence

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https://doi.org/10.1016/j.diagmicrobio.2017.09.014

in the 23S rDNA with the Lightmix® hybridization probe via melting curve analysis. As known from previous studies, resistance levels vary depending on the mutation in the 23S rDNA, permitting an evaluation of treatment outcome depending on the detected point mutation. More precisely, A2142G point mutations are related to higher (> 64 mg/L) minimum inhibitory concentrations (MICs) and lower (< 64 mg/L) MICs are often associated with A2143G mutations, whereas A2142C point mutations are rarely found and thus no statement can be made on the CLR resistance level they convey (van Doorn et al., 2001; Versalovic et al., 1997).

Besides the detection of *H. pylori* and the identification of CLR resistance in biopsies, which can just be retrieved from patients via an invasive endoscopy, *H. pylori* can be detected in stool specimens using RT-PCR. Evaluation from various studies using RT-PCR in stool samples showed exceptional specificity values, but sensitivity was rather low ranging from 62.5% to 73.0% (Falsafi et al., 2009; Lottspeich et al., 2007; Schabereiter-Gurtner et al., 2004; Şen et al., 2005).

The aim of this study was to compare CLR susceptibility testing by E®-Test and the Lightmix® RT-PCR using a set of 60 *H. pylori* strains showing phenotypical CLR susceptibility or CLR resistance (Minimum inhibitory concentrations from 0.016 to 256 mg/L). Furthermore, we evaluated *H. pylori* and CLR resistance detection in clinical specimens (biopsy and stool) by Lightmix® RT-PCR.

2. Materials and methods

2.1. H. pylori strains used in the study

In this study a set of *H. pylori* strains (N = 60) from the culture collection of the Institute of Medical Microbiology, University of Zurich, were used. These strains were isolated between 2013 and 2015 from gastric biopsies that were sent to the laboratory for susceptibility testing after CLR treatment failure. The strains were intentionally selected based on their phenotypic resistance status to get an evenly distributed number of CLR susceptible (MIC ≤0.25 mg/L) and CLR resistant strains (0.5 < MIC). After thawing, the isolates were incubated on Brucella Agar plates (Becton Dickinson, Allschwil, Switzerland) for 3 days at 37 °C in a microaerophilic atmosphere (90% N_2, 5% CO_2, 5% O_2) using a gas generator (CampyGen; Oxoid, Basingstoke, England). Subcultures were made from the *H. pylori* isolates after 3 days to obtain enough biomass for the E-Test® (bioMérieux, Marcy l'Etoile, France) and to perform DNA extraction using InstaGene®. Two colonies from each bacterial subculture were suspended in 0.85% NaCl. DNA was extracted using InstaGene® (Bio-Rad, Hercules, CA, USA), following the manufacturer's recommendations with the following modifications: centrifugation for 10 minutes instead of 1 minute at 10′000 g before removal of the supernatant, incubation at 56 °C for two hours instead of 30 minutes, and incubation for 10 instead of 8 minutes at 96 °C instead of 100 °C. All *H. pylori* strains (N = 60) were analyzed by E-Test® and by Lightmix® RT-PCR (TIBMolbiol, Berlin, Germany).

2.2. Phenotypical CLR susceptibility testing by E-Test®

The *H. pylori* culture was adjusted to a McFarland Standard of 3 (McFarland, 1907). A rapid urease test (Becton Dickinson) was performed with each *H. pylori* strain, following the producer's recommendations, before carrying out the E-Test® to confirm a successful *H. pylori* growth and exclude contamination. A stripe containing CLR (bioMérieux, 0.016–256 mg/L) was put on a Mueller Hinton agar plate with horse blood (Becton Dickinson). The plates were incubated in a microaerobic atmosphere at 37 °C for 3 days. Subsequently, the MIC was determined using a light microscope (Leica M80, Leica Microsystems). Susceptibility interpretation was performed, according to EUCAST (EUCAST, 2016), applying a clinical breakpoint of ≤0.25 mg/L for CLR susceptible and >0.5 mg/L for CLR resistant strains.

2.3. Lightmix® RT-PCR assay and melting curve analysis

For the RT-PCR, the extracted DNA was added to a mixture consisting of Roche PCR-grade water (Roche, Rotkreuz, Switzerland), a LightCycler® DNA multiplex master mix (Roche) and the Lightmix® primers and probes (TIBMolbiol). We followed the RT-PCR mastermix composition guidelines and the LightCycler® (LC) amplification protocol provided by TIBMolbiol. The RT-PCR was performed on a LightCycler480-II® (Roche). The RT-PCR amplification protocol was followed by a melting curve analysis on the LightCycler480-II® instrument, following the LC protocol from TIBMolbiol.

2.4. Performance of the Lightmix® RT-PCR assay

A dilution series (from 10^9 to 10^3 CFUs/ml) of 4 *H. pylori* cultures (wild-type, A2142G, A2142C and A2143G genotype) was done in 0.85% NaCl to determine the sensitivity of *H. pylori* detection by Lightmix® RT-PCR. DNA was extracted from each dilution using InstaGene® as stated above and analyzed by Lightmix® RT-PCR and melting curve analysis.

To determine the specificity of the Lightmix® primers and probes, we chose a set of commensal and pathogenic bacteria that may occur in clinical specimens (Table 1). These bacterial isolates were obtained from the strain collection of the Institute of Medical Microbiology and cultured on COS blood agar or McConkey agar plates (BD) at 37 °C.

Table 1
Bacterial strains isolated from clinical specimens used for determining the specificity of the *H. pylori* Lightmix® RT-PCR.

Sample_ID	Species		Material	RT-PCR Result
	Camplyobacter spp.			
2016112726		*C. jejuni*	stool	+
2016112690		*C. jejuni*	stool	+
2016112682		*C. coli*	stool	+
50205		*C. fetus*	stool	+
	Corynebacterium spp.			
51573		*C. amycolatum*	culture	-
5063		*C. propinquum*	culture	-
48036		*C. imitans*	culture	-
1532		*C. pseudodiphtheriticum*	culture	-
	Escherichia sp.			
52289		*E. coli*	culture	-
	Salmonella spp.			
48504		*S. enterica*	culture	-
1657465		*S. typhimurium*	culture	-
	Staphylococcus spp.			
39614		*S. aureus*	culture	-
53962		*S. caprae*	culture	-
53440		*S. epidermidis*	culture	-
54607		*S. gallinarum*	culture	-
54669		*S. haemolyticus*	culture	-
42000		*S. hominis*	culture	-
53225		*S. lugdunensis*	culture	-
53222		*S. pettenkoferi*	culture	-
52557		*S. pasteuri*	culture	-
	Streptococcus spp.			
39702		*S. anginosus*	culture	-
3685		*S. constellatus*	culture	-
47158		*S. bovis*	culture	-
44666		*S. gallolyticus*	culture	-
3846		*S. gordonii*	culture	-
19733		*S. intermedius*	culture	-
24657		*S. mitis*	culture	-
30611		*S. mutans*	culture	-
48502		*S. oralis*	culture	-
3290		*S. salivarius*	culture	-

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DNA was extracted using InstaGene® as stated above and analyzed by Lightmix® RT-PCR.

2.5. Sequencing of the 23S rDNA

To verify the detected 23 rDNA sequence, all RT-PCR products were sequenced. After RT-PCR, the amplification products were purified using the QIAquick PCR Purification Kit (QIAGEN), following the producer's recommendations. The BigDye® sequencing kit solutions as well as the *H. pylori* primers were added to our template DNA. A Veriti™ Thermal Cycler (Applied Biosystems, Forster City, CA, USA) was used for the cycle sequencing program. This program included an initial denaturation of the templates at 96 °C for 1 minute, followed by 25 cycles that each consisted of a denaturation at 96 °C for 10 seconds, and an annealing and elongation step for 1 minute at 60 °C. The cycle sequencing products were then cleaned using Performa® DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, CA, USA) and centrifuged for 2 minutes at 750 rpm. The cleaned amplification products were sequenced on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems).

2.6. Clinical biopsy and stool specimens used in the study

H. pylori was cultivated from fresh biopsies (N = 6) that were sent to our institute. Subculturing was not necessary as enough biomass was obtained to perform the E-Test®. For the Lightmix® RT-PCR, DNA was extracted from the biopsy using the EZ1® DNA Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. Results from *H. pylori* and CLR resistance detection by E-Test® and by Lightmix® RT-PCR were compared for the 6 gastric biopsy specimens.

To determine the sensitivity of *H. pylori* detection in stool specimens, a dilution series (from 10^9 to 10^2 CFUs/ml) was done with 3 *H. pylori* cultures (wild-type, A2142G and A2143G genotype); 100 μL of each dilution was spiked into 900 μL of liquid stool and DNA was extracted from the spiked stool samples with the PSP® spin stool DNA kit (Invisorb Stratec, Berlin, Germany), following the producer's recommendations. *H. pylori* and CLR resistance was detected by Lightmix® RT-PCR and melting curve analysis. To determine the specificity of *H. pylori* detection in stool samples, four *Campylobacter* sp. positive stool samples were processed with the PSP® spin stool DNA kit (Invisorb Stratec, Berlin, Germany), following the producer's recommendations, and analyzed by Lightmix® RT-PCR.

2.7. Ethics

The research was conducted in accordance with the Declaration of Helsinki and national and institutional standards.

3. Results

3.1. Performance of the H. pylori Lightmix® RT-PCR

The *H. pylori* Lightmix® RT-PCR detected 10^9 to 10^3 23S rRNA gene copies per ml and showed a clear melting peak independent of gene copy number (Fig. S1). Melting curve analysis showed clear melting temperature (T_m) shifts between the wild-type strains and strains showing nucleotide mismatches with the 23S rDNA probe. The *H. pylori* wild-type showed a distinct T_m at 63 °C, whereas the mutation variants A2142C, A2143G and A2142G showed a T_m at 54 °C, 56 °C and 59 °C, respectively (Fig. S1).

3.2. CLR resistance detection by E-Test® and Lightmix® RT-PCR using H. pylori strains

We determined the MIC of CLR by E-Test® for 60 *H. pylori* strains (Fig. 1, Table S1). Accordingly, 20/60 strains were classified as CLR susceptible (MIC ≤0.25 mg/L) and 40/60 strains were classified as CLR resistant (0.5 < MIC). All 20 *H. pylori* strains that were determined as CLR susceptible by E-Test® showed a wild-type genotype at a T_m of 63 °C in melting curve analysis (Fig. 2a). Among the 15 CLR resistant strains with a 0.5 < MIC <256, 13/15 showed a T_m at 56 °C (A2143G) and 2/15 a T_m at 63 °C (Fig. 2b) like the CLR susceptible wild-type strains. The two *H. pylori* strains with a T_m at 63 °C had a MIC of 48 and 128 mg/L. 23S rDNA sequencing confirmed the wild-type genotype for both *H. pylori* isolates (Fig. 1, Table S1). Among the 25 CLR resistant *H. pylori* strains with MIC ≥256, 1/25 showed a T_m at 54 °C (A2142C), 9/25 a Tm at 56 °C (A2143G) and 14/25 showed a T_m at 59 °C (A2142G) (Fig. 2c). There was one heterozygous strain that carried two different point mutations (A2142G and A2143G), one in each 23S rRNA gene. One strain showed a T_m at 63 °C, which infers a wild-type genotype, though a MIC ≥256 was detected by E-Test®.

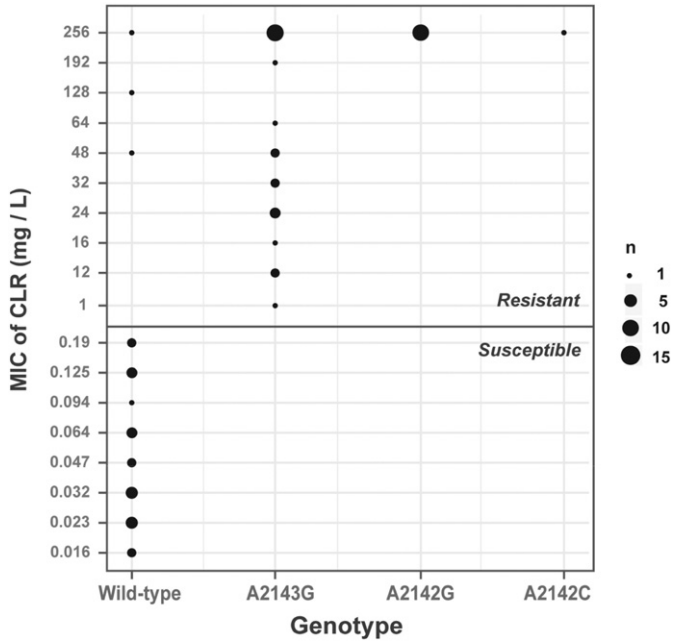

Fig. 1. Relationship between phenotypical CLR susceptibility testing by E-Test® (≤0.25 mg/L relates to CLR susceptible; >0.5 mg/L relates to CLR resistant) and the detected *H. pylori* genotypes in melting curve analysis using 60 *H. pylori* strains.

Discrepant results between E-Test® and RT-PCR were obtained for 3 *H. pylori* strains that showed a CLR resistant phenotype, whereas a T_m of 63 °C was detected in melting curve analysis, indicating a CLR susceptible *H. pylori* wild-type. The wild-type sequence was confirmed by 23S rDNA sequencing for all 3 *H. pylori* isolates (Fig. 1, Table S1). Overall, a concordance of the two methods was found in 57 of the 60 samples (95%) (Table 2). This coincides with previous studies that showed concordance rates between E-Test® and laboratory-developed PCR assays ranging from 71.2% to 98.4% (Chisholm and Owen, 2008; Lascols et al., 2003; Oleastro et al., 2003).

3.3. CLR resistance detection by E-Test® and Lightmix® RT-PCR using H. pylori isolated from gastric biopsies

We determined the MIC of CLR by E-Test® for 6 *H. pylori* strains isolated from gastric biopsies (Table 3). 1/6 strains were classified as CLR susceptible (MIC ≤0.25 mg/L) and 5/6 strains were classified as CLR resistant (0.5 < MIC). 2/6 showed a Tm at 56 °C (A2143G) and 2/6 showed a T_m at 59 °C (A2142G). Two *H. pylori* strains showed a wild-type genotype at a T_m of 63 °C, though one was determined as CLR susceptible (0.032 mg/L) and one as CLR resistant (1 mg/L) by E-Test®. 23S rDNA sequencing confirmed the wild-type sequence for the *H. pylori* isolate (Table 3).

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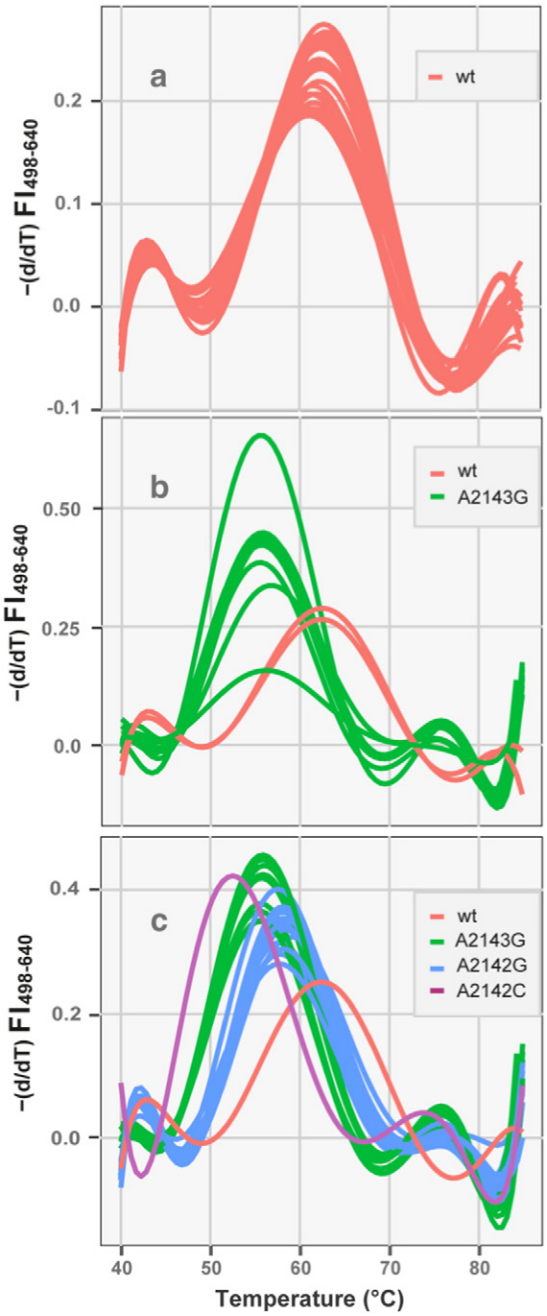

Fig. 2. Melting curve analysis of 60 *H. pylori* strains showing phenotypical CLR susceptibility with MIC ≤0.25 mg/L (a); CLR resistance with 0.5 < MIC <256 mg/L (b), and CLR resistance with MIC ≥256 mg/L (c).

3.4. Detection of H. pylori in stool specimens

Stool samples were spiked with 3 different *H. pylori* isolates (wild-type, A2142G and A2143G genotype). Stool samples with initial concentrations of 10^9–10^4 CFUs/ml before DNA extraction showed a clear amplification signal in the *H. pylori* RT-PCR (Fig. 3a) and melting curve analysis identified the correct *H. pylori* genotype (Fig. 3b).

Campylobacter jejuni and *Campylobacter fetus* showed a late amplification signal in the *H. pylori* RT-PCR (from the 35th cycle on) with an amplification delay of 10 cycles compared to the same amount of *H. pylori* DNA, and gave a melting peak with a T_m at 63 °C in melting curve analysis.

Table 2
Results from CLR resistance detection with the E-Test® and the Lightmix® *H. pylori* RT-PCR in *H. pylori* strains (N = 60).

		E-Test®	
		Susceptible to CLR	Resistant to CLR
RT-PCR	Susceptible to CLR	20	3
	Resistant to CLR	0	37

4. Discussion

Rapid detection and accurate identification of *H. pylori* and CLR resistance genes is crucial for proper antibiotic therapy, and to monitor resistance epidemiology. Commercially available tests have several advantages over laboratory-developed assays, i.e., production is highly standardized, quality is controlled by the manufacturer, reagents are ready to use, and the shelf-life of the test kits is guaranteed. Several in-house RT-PCR assays for the genetic detection of *H. pylori* and CLR resistance were previously published (e.g., 19). The LightMix® *H. pylori* kit is a new, commercially available RT-PCR assay for the detection of *H. pylori* and CLR resistance from cultures and biopsies. The limit of detection of the Lightmix® RT-PCR was found at 10^3 CFUs/ml, which corresponds well with the assay information provided by TIBMolbiol. Melting curve analysis always detected the correct *H. pylori* genotype independent of the 23S rRNA gene copy number present in the assay. Our results showed that different point mutations are associated with different levels of CLR resistance as the A2142G and A2142C point mutations were always related to high CLR resistance levels (MIC ≥256 mg/L).

In contrast, phenotypical susceptibility testing is very time consuming (10 to 14 days) by conventional methods (e.g., E-Test®) and presents challenges due to *H. pylori's* slow growth and its fastidious growth requirements. Moreover, we observed in our study that it is not always possible to determine an explicit MIC of CLR with the E-Test®, because of the small and transparent colonies that *H. pylori* forms and the inter-assay variability depending on the analyst performing the E-Test®. Furthermore, low bacterial loads after eradication treatment and overgrowth of contaminating microorganism can reduce the sensitivity of cultural testing methods (Chisholm and Owen, 2008).

We found high concordance (95%) for *H. pylori* and CLR resistance detection in cultures and biopsies by E-Test® and Lightmix® RT-PCR. Four bacterial isolates showed a CLR resistant phenotype determined by E-Test® and a CLR susceptible genotype detected by Lightmix® RT-PCR. 23S rDNA sequencing confirmed the CLR susceptible genotype detected in the Lightmix® RT-PCR and melting curve analysis. Discrepant results between phenotypical and genotypical CLR susceptibility screening may be explained by the fact that the Lightmix® *H. pylori* specific probe covers the 3 most prevalent point mutations in the 23S rRNA gene (A2142G, A2143G and A2142C), but not all rarely occurring ones (e.g. point mutation C2182T) (Chen et al., 2008). Except for 23S rRNA mutations, expression of an active drug efflux mechanism, responsible for rapidly transferring clarithromycin out of the bacterial cell, and preventing the binding of the antibiotic to the ribosome, may play an important role in acquired CLR resistance (Hirata et al., 2010; Webber and Piddock, 2003).

Detection of *H. pylori* in stool specimens was very accurate and the correct genotype was characterized in melting curve analysis for all *H. pylori* isolates. The limit of detection was found at 10^4 DNA copies per ml that corresponds well with previous studies (Schabereiter-Gurtner et al., 2004). However, *C. jejuni* and *C. fetus* produced unspecific positive results if present in high concentrations. We therefore decided to complement the *H. pylori* RT-PCR with a CE-IVD labeled genus specific probe for *Campylobacter* (TIBMolbiol), and always used duplex RT-PCRs for *H. pylori* detection in clinical specimens. This allowed us to detect *Campylobacter* spp. when present in clinical samples (biopsy and feces), and avoid false positive results for *H. pylori* detection.

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Table 3
Summary of the results from the E-Test®, the Lightmix® RT-PCR and the 23S rDNA sequencing from 6 *H. pylori* strains isolated from gastric biopsies.

Sample_ID	Species	Material	E-Test®	MIC of CLR (mg L^{-1})	RT-PCR	Melt curve T_m	Genotype
2016101014	*H. pylori*	biopsy	+	0.032	+	63 °C	wild-type
2016100888	*H. pylori*	biopsy	+	1	+	63 °C	wild-type
2016101038	*H. pylori*	biopsy	+	8	+	56 °C	A2143G
2016100928	*H. pylori*	biopsy	+	96	+	56 °C	A2143G
2016100948	*H. pylori*	biopsy	+	>256	+	59 °C	A2142G
2016101035	*H. pylori*	biopsy	+	>256	+	59 °C	A2142G

In conclusion, our data showed that there is a strong association between specific mutations in the 23S rDNA and CLR resistance. The RT-PCR proved to be an exceptional tool for a fast and reliable detection of *H. pylori*. Furthermore, it allows for CLR resistance screening within a few hours prior to the prescription of an antibiotic therapy and should drastically reduce the cases of treatment failure as CLR is just administered if no resistance is detected. These improved detection rates and antibiotic susceptibility information is especially important when *H. pylori* culture is unsuccessful.

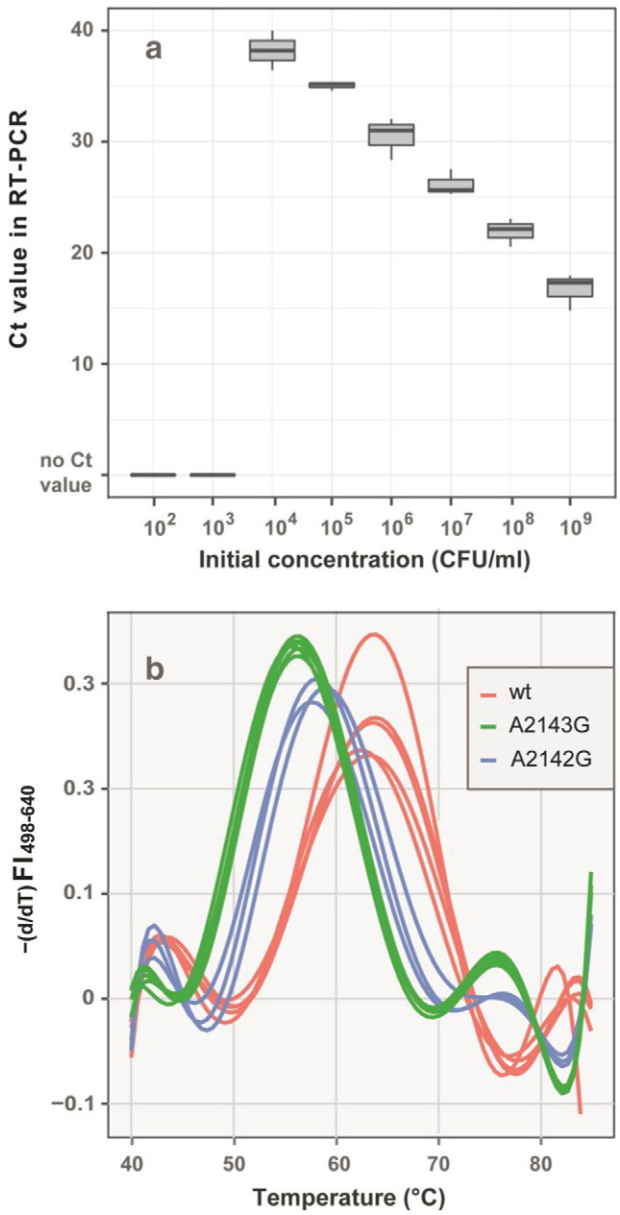

Fig. 3. RT-PCR detection of 3 *H. pylori* isolates (wild-type, A2142G and A2143G genotype) that were spiked into stool specimens; initial concentration refers to the CFU/ml in the stool sample before DNA extraction (a) and the results from the subsequent melting curve analysis (b).

Acknowledgements

We thank the technicians, especially Blasowitsch Susanne, of the Institute of Medical Microbiology for expert help and assistance. We thank the Institute of Medical Microbiology, University of Zurich, for continuous support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2017.09.014.

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Please cite this article as: Redondo JJ, et al, A novel RT-PCR for the detection of *Helicobacter pylori* and identification of clarithromycin resistance mediated by mutations in t..., Diagn Microbiol Infect Dis (2017), https://doi.org/10.1016/j.diagmicrobio.2017.09.014